RECOMBINANT PROTEIN CONTAINING A C-TERMINAL FRAGMENT OF *PLASMODIUM* MSP-1

The invention relates to novel active principles for vaccines derived from the major surface protein in merozoite forms of a *Plasmodium* which is infectious for mammals, especially humans, more generally termed MSP-1.

Description of the Background

MSP-1 has already been the subject of a number of studies. It is synthesized in the schizont stage of *Plasmodium* type parasites, in particular *Plasmodium falciparum*, and is expressed in the form of one of the major surface constituents of merozoites both in the hepatic stage and in the erythrocytic stage of malaria (1, 2, 3, 4). Because of the protein's predominant character and conservation in all known *Plasmodium* species, it has been suggested that it could be a candidate for constituting anti-malarial vaccines (5, 6).

The same is true for fragments of that protein, particularly the natural cleavage products which are observed to form, for example during invasion by the parasite into erythrocytes of the infected host. Among such cleavage products are the C-terminal fragment with a molecular weight of 42 kDa (7, 8) which is itself cleaved once more into an N-terminal fragment with a conventional apparent molecular weight of 33 kDa and into a C-terminal fragment with a conventional apparent molecular weight of 19 kDa (9) which remains normally fixed to the parasite membrane after the modifications carried out on it, via glycosylphosphatidylinositol (GPI) groups (10, 11).

It is also found at the early ring stage of the intraerythrocytic development cycle (15, 16), whereby the observation was made that the 19 kDa fragment could play a role which is not yet known, but which is doubtless essential in re-invasive processes. This formed the basis for hypotheses formed in the past that that protein could constitute a particularly effective target for possible vaccines.

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It should be understood that the references frequently made below to the p42 and p19 proteins from a certain type of *Plasmodium* are understood to refer to the corresponding C-terminal cleavage products of the MSP-1 protein of that *Plasmodium* or, by extension, to products containing substantially the same amino acid sequences, obtained by genetic recombination or by chemical synthesis using conventional techniques, for example using the "Applied System" synthesizer, or by "Merrifield" type solid phase synthesis. For convenience, references to "recombinant p42" and "recombinant p19" refer to "p42" and "p19" obtained by techniques comprising at least one genetic engineering step.

Faced with the difficulty of obtaining large quantities of parasites for *P. falciparum* and the impossibility of cultivating *P. vivax in vitro*, it has become clear that the only means of producing an anti-malaria vaccine is to resort to techniques which use recombinant proteins or peptides. However, MSP-1 is very difficult to produce whole because of it large size of about 200 kDa, a fact which has led researchers to study the C-terminal portion, the (still unknown) function of which is probably the more important. In addition, the extensive polymorphism in the N-terminal portion of MSP-1 has negative implications for the use of these parts of the molecule in vaccine preparation.

Recombinant proteins concerning the C-terminal portion of the *P. falciparum* MSP-1 which have been produced and tested in the monkey (12, 40, 41) are:

- a p19 fused with a glutathione-S-transferase produced in *E. coli* (40);
- a p42 fused with a glutathione-S-transferase produced in *E. coli* (12);
- a p19 fused with a polypeptide from a tetanic anatoxin and carrying auxiliary T cell epitopes produced in *S. cerevisiae* (12);
- a p42 produced in a baculovirus system (41).

A composition containing a p19 protein fused with a glutathione-S-transferase produced in *E. coli* combined with alum or liposomes did not

exhibit a protective effect in any of six vaccinated *Aotus nancymai* monkeys (40).

A composition containing a p42 protein fused with a glutathione-S-transferase produced in *E. coli* combined with Freunds complete adjuvant did not exhibit a protective effect in two types of *Aotus* monkeys (*A. nancymai* and *A. vociferans)* when administered to them. The p19 protein produced in *S. cerevisiae* exhibited a protective effect in two *A. nancymai* type *Aotus* monkeys (12). In contrast, there was no protective effect in two *A. vociferans* type *Aotus* monkeys.

Some researchers (18) have also reported immunization tests carried out in the rabbit using a recombinant p42 protein produced in a baculovirus system and containing one amino acid sequence in common with *P. falciparum* (18). Thus these latter authors indicate that in the rabbit that recombinant p42 behaves substantially in the same way as the entire recombinant MSP-1 protein (gp195). This p42 protein in combination with Freunds complete adjuvant has been the subject matter of a vaccination test in a non-human primate susceptible to infection by *P. falciparum*, *Aotus, lemurinus grisemembra* (40). The results showed that 2 of 3 animals were completely protected and the third, while exhibiting a parasitemia which resembled that of the controls, had a longer latent period. It is nevertheless risky to conclude to a protective nature in man of the antibodies thus induced against the parasites themselves. It should be remembered that there are currently no very satisfactory experimental models in the primate for *P. vivax* and *P. falciparum*. The *Saimiri* model, developed for *P. falciparum* and *P. vivax*, and the *Aotus* model for *P. falciparum*, are artificial systems requiring the parasite strains to be adapted and often requiring splenectomy of the animals to obtain significant parasitemia. As a result, the vaccination results from such models can only have a limited predictive value for man.

In any event, what the real vaccination rate would be which could possibly be obtained with such recombinant proteins is also questionable, bearing in mind the discovery - reported below - of the presence in p42s from *Plasmodiums* of the same species, and more particularly in the corresponding p33s, of hypervariable regions which would in many cases render uncertain the immunoprotective efficacy of antibodies induced in individuals vaccinated with a p42 from a *Plasmodium* strain against an infection by other strains of the same species (13).

It can even be assumed that the high polymorphism of the N-terminal portion of p42 plays a significant role in immune evasion, often observed for that type of parasite.

SUMMARY OF THE INVENTION

The aim of the present invention is to produce vaccinating recombinant proteins which can escape these difficulties, the protective effect of which is verifiable in genuinely significant experimental models or even directly in man.

BRIEF DESCRIPTION OF THE DRAWING

More particularly, the invention provides vaccinating compositions against a *Plasmodium* type parasite which is infectious for man, containing as an active principle a recombinant protein which may or may not be glycosylated, whose essential constituent polypeptide sequence is:

- either that of a 19 kilodalton (p19) C-terminal fragment of the surface protein 1 of the merozoite form (MSP-1 protein) of a *Plasmodium* type parasite which is infectious for man, said C-terminal fragment remaining normally anchored to the parasite surface at the end of its penetration phase into human erythrocytes in the event of an infectious cycle;
- or that of a portion of that fragment which is also capable of inducing an immune response which can inhibit *in vivo* parasitemia due to the corresponding parasite;
- or that of an immunologically equivalent peptide of said p19 fragment or said portion of that fragment; and

said recombinant protein further comprises conformational epitopes which are unstable in a reducing medium and which constitute the majority of the epitopes recognized by human antiserums formed against the corresponding *Plasmodium*.

The presence of such conformational epitopes plays an important role in the protective efficacy of the active principle of the vaccines. They are particularly found in the active principles which exhibit the other characteristics defined above, when they are produced in a baculovirus system. If need be, it is mentioned below that the expression "baculovirus vector system" means the ensemble constituted by the baculovirus type vector itself and the cell lines, in particular cells of insects transfectable by a baculovirus modified by a sequence to be transferred to these cell lines resulting in expression of that transferred sequence. Preferred examples of these two partners in the baculovirus system have been described in the article by Longacre et al. (14). The same system was used in the examples below. It goes without saying, of course, that variations in the baculovirus and in the cells which can be infected by the baculovirus can be used in place of those selected.

In particular, the recombinant protein is recognized by human antiserums formed against the corresponding *Plasmodium* or against a homologous *Plasmodium* when it is in its non reduced state or in a reduced non irreversible state, but is not recognized or is only recognized to a slight extent by these same antiserums when it is irreversibly reduced.

The unstable character of these conformational epitopes in a reducing medium can be demonstrated by the test described below in the examples, in particular in the presence of ß-mercaptoethanol. Similarly, the examples below describe the experimental conditions applicable to obtain irreversible reduction of the proteins of th invention.

From this viewpoint, the recombinant protein produced by Longacre et al. (14) can be used in such compositions. It should be remembered that S. Longacre et al. succeeded in producing a recombinant p19 from the MSP-1 of *P. vivax* in a baculovirus vector system containing a nucleotide sequence coding for the p19 of *Plasmodium vivax*, in particular by transfecting cultures of insect cells [*Spodoptera frugiperda (Sf9)* line] with baculovirus vectors containing, under the control of the polyhedrin promoter, a sequence coding for the peptide sequences defined below, with the sequences being placed in the following order in the baculovirus vector used:

- a 35 base pair 5' terminal fragment of the polyhedrin signal sequence, in which the methionine codon for initiating expression of this protein had been mutated (to ATT);
- a 5'-terminal nucleotide fragment coding for a 32 amino acid peptide corresponding to the N-terminal portion of MSP-1, including the MSP-1 signal peptide;
- either a nucleotide sequence coding for p19, or a sequence coding for the p42 of the MSP-1 protein of *Plasmodium vivax*, depending on the case, these sequences also being provided with ("anchored" forms) or deprived of (soluble forms) 3' end regions of these nucleotide sequences, whose end C-terminal expression products are reputed to play an essential role in anchoring the final p19 protein to the parasite membrane;
- 2 TAA stop codons.

For p42, the sequences derived from the C-terminal region of MSP-1 extend consequently from amino acid Asp 1325 to amino acid Leu 1726 (anchored form) or to amino acid Ser 1705 (soluble form) and for p19, the sequences extend from amino acid Ile 1602 to amino acid Leu 1726 (anchored form) or to amino acid Ser 1705 (soluble form) it being

understood that the complete amino acid sequences of p42 and p19, whose initial and terminal amino acids have been indicated above follow from the gene of the Belem isolate of *P. vivax* which has been sequenced (20).

Similar results were obtained using, in the same vector systems, nucleotide sequences coding for the p42 and p19 of *Plasmodium cynomolgi*. The interest in *P. cynomolgi* is twofold: it is a parasitic species very close to *P. vivax* which is infectious for the macaque. It can also infect man. Furthermore, a natural host of *P. cynomolgi*, the toque macaque, is accessible for testing the efficacy of the protection of MSP-1 from *P. cynomolgi* in a natural system. In addition, the rhesus monkey which is considered to be one of the most representative species for immune reactions in man, can also be infected by *P. cynomolgi*.

In particular, excellent results have been obtained in vaccination tests carried out using the toque macaque with two recombinant polypeptides: soluble p42 and, in particular, soluble p19 derived from *P. cynomolgi*, respectively produced in a baculovirus system and purified on an affinity column with monoclonal antibodies recognizing the corresponding regions of the native MSP-1 protein. The following observations were made: six monkeys immunized with only p19 (three monkeys) and the p19 and p42 together (three monkeys) all exhibited practically sterile immunity after challenge infection. The results obtained in the three monkeys immunized with p42 were less significant. Two of them were as above, but since the third exhibited a lower parasitemia than the controls immunized with a PBS buffer in the presence of Freunds adjuvant (3 monkeys) or not immunized (3 monkeys), it was less clear.

A second challenge infection showed that the monkeys which had received p19 alone were protected for at least six months. A second vaccination test with p19 in combination with alum in this system (toque

macaque *P. cynomolgi*) exhibited significant protection for 2 of the 3 monkeys. This is the first time that MSP-1 or another recombinant antigen has demonstrated a protective effect in the presence of alum (42).

The particularly effective test results carried out with the macaque with recombinant polypeptides produced in a baculovirus system using a recombinant p19 from *P. cynomolgi* showed that recombinant polypeptides respectively containing recombinant p19s from other *Plasmodiums* must behave in the same manner. They are more meaningful for malaria in man than the results from tests carried out with *P. vivax* or *P. falciparum* in their "artificial hosts".

Baculovirus recombinant proteins derived from a C-terminal MSP-1 portion (p19) have a very significant antimalarial protective effect in a natural system, which constitutes the most representative model for evaluating the protective effect of MSP-1 for man.

The protective effect obtained can be further improved if the p19 form is deprived of the hypervariable region of the N-terminal portion of p42, the effect of which can be deleterious in natural situations in which the vaccinated subject is confronted by a great deal of polymorphism. Further, p19 appears to possess specific epitopes which are not present in p42 (Holm et al., 1997).

The 19 kDa C-terminal fragment, the sequence of which is present in the active principle of the vaccine, can be limited to the sequence for the p19 itself, in the absence of any polypeptide sequence normally upstream of the p19 sequence in the corresponding MSP-1 protein. Clearly, though, the essential constituent polypeptide sequence of the active principle can also comprise a polypeptide sequence for the C-terminal side belonging to the 33 kDa (p33) N-terminal fragment still associated with the p19 in the corresponding p42, before natural cleavage of the latter, if the pr s nce of this fragment does not modify the immunological properties of the active

principle of the vaccine. As will be seen below, in particular in the description of the examples, the C-terminal sequences of the p33 in various strains of the same species of *Plasmodium* (see the C-terminal portion of the peptide sequences of "region III" in Figure 4 (SEQ ID NOS: 11-14)) also have a degree of homology or substantial conservation of the sequence, for example on the order of at least 80%, in different varieties of *Plasmodiums* which are infectious for man, such that they do not fundamentally modify the vaccinating properties of the active principle (the sequence of which corresponds to region IV in Figure 4), in particular using the hypothesis which follows from this figure; that the presumed cleavage site between the p19 and region III of the p33 is located between the leucine and asparagine residues in a particularly well conserved region (LNVQTQ).

Normally the C-terminal polypeptide sequence of the p33, when it is present, comprises less than 50 amino acid residues, or even less than 35, preferably less than 10 amino acid residues.

The essential constituent polypeptide sequence of the active principle of the vaccine need not comprise all of the sequence coding for p19, naturally providing that the latter retains the ability to induce antibodies which protect against the parasite. Preferably, this polypeptide fragment portion contains the two EGF (Epidermal Growth Factor) regions.

Clearly, the skilled person could distinguish between active fragments and those which would no longer be so, in particular experimentally by producing modified vectors containing inserts with different lengths originating from the p19, respectively isolated from the fragments obtained from the sequence coding for p19, by reaction with appropriate restriction enzymes, or by exonucleolytic enzymes which would be kept in contact with the fragment coding for p19 for differing periods; the capacity of the expression products from these inserts in the corresponding eukaryotic cells, in particular in insect cells, transformed by the corresponding modified

vectors, to exert a protective effect can then be tested, in particular under the experimental conditions which are described below in the examples. In particular, the expression products of these inserts must be able to inhibit a parasitemia induced *in vivo* by the corresponding whole parasite.

Thus, the invention includes all vaccinating compositions in which the essential constituent polypeptide sequence of the active principle is constituted by a peptide which can induce a cellular and/or humoral type immunological response equivalent to that produced by p19 or a fragment as defined above, provided that the addition, deletion or substitution in the sequence of certain amino acids by others would not cause a large modification of the capacity of the modified peptide - hereinafter termed the "immunologically equivalent peptide" - to inhibit said parasitemia.

The p19 fragment can naturally also be associated at the N-terminal side or the C-terminal side or via a peptide bond to a further plasmoidal protein fragment having a vaccinating potential (such as Duffy binding protein from *P. vivax* (29) or EBA-175 from *P. falciparum* (30) and (31), one region of which is specifically rich in cysteine), provided that its capacity to inhibit parasitemia normally introduced *in vivo* by the corresponding parasite is not altered but is amplified.

Upstream of the N-terminal end of p19, the fragment coding for p19 or a portion thereof can also contain a peptide sequence which is different again, for example a C-terminal fragment of the signal peptide used, such as that for the MSP-1 protein. This sequence preferably comprises less than 50 amino acids, for example 10 to 40 amino acids.

These observations pertain in similar fashion to the p19 from other *Plasmodia*, in particular *P. falciparum*, the dominant species of the parasite, responsible for the most serious forms of malaria.

However, the techniques summarized above for producing a recombinant p19 from *P. vivax* or *P. cynomolgi* in a baculovirus system are

difficult to transpose unchanged to producing a recombinant p19 of *P. falciparum* in a satisfactory yield, if only to obtain appreciable quantities which will allow immunoprotective tests to be carried out.

The invention also provides a process which overcomes this problem to a large extent. It also becomes possible to obtain much higher yields of *P. falciparum* p19 - and other *Plasmodiums* where similar difficulties are encountered - using a synthetic nucleotide sequence substituting the natural nucleotide sequence coding for the p19 of *Plasmodium falciparum* in an expression vector of a baculovirus system, this synthetic nucleotide sequence coding for the same p19, but being characterized by a higher proportion of G and C nucleotides than in the natural nucleotide sequence.

In other words, the invention follows from the discovery that expression of a nucleotide sequence coding for a p19 in a baculovirus system is apparently linked to an improved compatibility of successive codons in the nucleotide sequence to express with the "cellular machinery" of the host cells transformable by the baculovirus, in the manner of that observed for the natural nucleotide sequences normally contained in these baculovirus and expressed in the infected host cells; hence the poor expression, or even total absence of expression of a native *P. falciparum* nucleotide sequence; hence also a possible explanation of the more effective expression observed by Longacre et al. (14) for the p19 of *P. vivax* in a baculovirus system and, as the inventors have also shown, of the *P. cynomolgi* sequence from corresponding native p19 nucleotide sequences, because of their relatively much higher amounts of G and C nucleotides than those of the native nucleotide sequences coding for the p19 of *P. falciparum*.

The invention thus more generally provides a recombinant baculovirus type modified vector containing, under the control of a promoter contained in said vector and able to be recognized by cells transfectable by

said vector, a first nucleotide sequence coding for a signal peptide exploitable by a baculovirus system, characterized by a second nucleotide sequence downstream of the first, also under the control of the promoter and coding for the peptide sequence:

- either of a 19 kilodalton (p19) C-terminal fragment of the surface protein 1 of the merozoite form (MSP-1 protein) of a *Plasmodium* type parasite other than *Plasmodium vivax* which is infectious for man, said C-terminal fragment remaining normally anchored to the parasite surface at the end of its penetration phase into human erythrocytes in the event of an infectious cycle;
- or of a portion of that peptide fragment provided that the expression product from the second sequence in a baculovirus system is also capable of inducing an immune response which can inhibit *in vivo* parasitemia due to the corresponding parasite;
- or of an immunologically equivalent peptide of said C-terminal peptide fragment (p19) or said peptide fragment portion by addition, deletion or substitution of amino acids not resulting in a large modification of the capacity of said immunologically equivalent peptide to induce a cellular and/or humoral type immunological response similar to that produced by said p19 peptide fragment or said portion of said fragment; and

said nucleotide sequence having, if necessary, a G and C nucleotide content in the range 40% to 60%, preferably at least 50%, of the totality of the nucleotides from which it is constituted. This sequence can be obtained by constructing a synthetic gene in which the natural codons have been changed for codons which are rich in G/C without modifying their translation (maintaining the peptide sequence).

The nucleotide sequence, provided by a synthetic DNA, may have at least 10% of modified codons with respect to the natural gene sequence or

cDNA while retaining the characteristics of the natural translated sequence, i.e., maintaining the amino acid sequence.

It is not excluded that this G and C nucleotide content could be further increased provided that the modifications resulting therefrom as to the amino acid sequence of the recombinant peptide - or immunologically equivalent peptide - produced do not result in a loss of immunological properties, or protective properties, of the recombinant proteins formed, in particular in the tests which will be described below.

These observations naturally apply to other *Plasmodium* which are infectious for man, in particular those where the native nucleotide sequences coding for corresponding p19s would have T and A nucleotide contents which are poorly compatible with effective expression in a baculovirus system.

The sequence coding for the signal used can be that normally associated with the native sequence of the *Plasmodium* concerned. But it can also originate from another *Plasmodium*, for example *P. vivax* or *P. cynomolgi* or another organism if it can be recognized as a signal in a baculovirus system.

The sequence coding for p19 or a fragment thereof in the vector under consideration is, in one case, deprived of the anchoring sequence of the native protein to the parasite from which it originates, in which case the expressed protein is generally excreted into the culture medium (soluble form). It is also remarkable in this respect that under the conditions of the invention, the soluble and anchored forms of the recombinant proteins produced, in particular when they are from *P. falciparum* or *P. cynomolgi* or *P. vivax*, tend to form oligomers, this property possibly being at the origin of the increased immunogenicity of the recombinant proteins formed.

The invention also concerns vectors in which the coding sequence contains the t rminal 3' end sequence coding for the hydrophobic C'-

terminal end sequence of the p19 which is normally implicated in the induction of anchoring the native protein to the cell membrane of the host in which it is expressed. This 3'-terminal end region can also be heterologous as regards the sequence coding for the soluble p19 portion, for example corresponding to the 3'-terminal sequence from *P. vivax* or from another organism when it codes for a sequence which anchors the whole of the recombinant protein produced to the cell membrane of the host of the baculovirus system used. An example of such anchoring sequences is the GPI of the CD59 antigen which can be expressed in the cells of *Spodoptera frugiperda* (32) type insects or the GPI of a CD14 human protein (33).

The invention also, naturally, concerns recombinant proteins, these proteins comprising conformational epitopes recognized by human serums formed against the corresponding *Plasmodium*.

In general, the invention also concerns any recombinant protein of the type indicated above, provided that it comprises conformational epitopes such as those produced in the baculovirus system, in particular those which are unstable in a reducing medium.

The invention also, naturally, concerns said recombinant proteins, whether they are in their soluble form or in the form provided with an anchoring region, in particular to cellular hosts used in the baculovirus system.

The invention also encompasses oligomers spontaneously produced in the baculovirus systems used or produced *a posteriori*, using conventional protein oligomerisation techniques. The most commonly used technique involves glutaraldehyde. However, any conventional system for bridging betwe n the respective amine and carboxyl functions in proteins can be used. As an example, any of th techniques described in European patent application EP-A-0 602 079 can be used.

The term "oligomer" means a molecule containing 2 to 50 monomer units, each of the monomer units containing p19 or a fragment thereof, as defined above, capable of forming an aggregate. The invention also encompasses any conjugation product between a p19 or a p19 fragment as defined above, and a carrier molecule - for example a polylysine-alanine - for use in producing vaccines, via bonds which are covalent or otherwise. The vaccinating compositions using them also form part of the invention.

The invention still further concerns vaccine compositions using these oligomeric or conjugated recombinant proteins, including proteins from *Plasmodium vivax*, these observations also extending to oligomers of these recombinant proteins.

The invention also encompasses compositions in which the recombinant proteins defined above are associated with an adjuvant, for example an alum. Recombinant proteins containing the C-terminal end region allowing them to anchor to the membrane of the cells in which they are produced are advantageously used in combination with lipids which can form liposomes appropriate to the production of vaccines. Without being limiting, lipids described, for example, in the publication entitled "Les liposomes aspects technologique, biologique et pharmacologique" [Liposomes: technological, biological and pharmacological aspects] by J. Delattre et al., INSERM, 1993, can be used.

The presence of the anchoring region in the recombinant protein, whether it is a homologous or heterologous anchoring region as regards the vaccinating portion proper, encourages the production of cytophilic antibodies, in particular IgG_{2a} and IgG_{2b} type in the mouse which could have a particularly high protective activity, so that associating the active principles of the vaccines so constituted with adjuvants other than the lipids used to constitute the liposome forms could be dispensed with. This amounts to a major advantage, since liposomes can be lyophilized under

conditions which enable them to be stored and transported, without the need for chains of cold storage means.

Other characteristics of the invention will become clear from the following description of examples of recombinant proteins of the invention and the conditions under which they can be produced. These examples are not intended to limit the scope of the invention.

Description of the construction of PfMSP1$_{p19}$S (soluble)

(soluble p19 from *P. falciparum*)

The recombinant construction PfMSP1$_{p19}$S contains the DNA corresponding to 8 base pairs of the leader sequence and the first 32 amino acids of the MSP-1 of *Plasmodium vivax* from Met$_1$ to Asp$_{32}$ (Belem isolate; Del Portillo et al., 1991, P. N. A. S., 88, 4030) followed by GluPhe due to the EcoR1 site connecting the two fragments. This is followed by the synthetic gene described in Figure 1, coding the *Plasmodium falciparum* MSP1$_{p19}$ from Asn$_{1613}$ to Ser$_{1705}$ (Uganda-Palo Alto isolate; Chang et al., 1988, Exp. Parasitol., 67, 1). The construction is terminated by two TAA stop codons. This construction gave rise to a recombinant protein which was secreted in the culture supernatant from infected cells.

In the same manner and for comparison, a recombinant construction was produced under conditions which were similar to those used to produce the p19 above, but working with a coding sequence consisting of a direct copy of the corresponding DNA of the *P. falciparum* strain (FUP) described by Chang et al., Exp. Parasit. 67,1; 1989. The natural gene copy (from asparagine 1613 to serine 1705) was formed from the native gene by PCR.

Figure 1A shows the sequences of both the synthetic gene (Bac19) (SEQ ID NO: 1) and the "native gene" (PF19) (SEQ ID NO: 3).

It can be seen that 57 codons of the 93 codons of the native sequence coding for the p19 from *P. falciparum* were modified (the third nucleotide in 55 of them and the first and third nucleotides in the other 2

codons). New codons were added to the 5' end to introduce the peptide signal under the conditions indicated above and to introduce an EcoRI site for cloning, and similarly two stop codons were added which were not present in the *P. falciparum* p19 to obtain expression termination signals. The individual letters placed above successive codons correspond to the respective successive amino acids. Asterisks (*) show the stop codons. Vertical lines indicate the nucleotides which are the same in the two sequences

Description of the PfMSP1$_{p19}$A construction (anchored GPI)

(anchored p19 of *P. falciparum*)

The PfMSP1$_{p19}$A construction had the characteristics of that above except that the synthetic sequence (**Figure 1B**) codes for the MSP1$_{p19}$ of *Plasmodium falciparum* (Uganda-Palo Alto isolate) from Asn$_{1613}$ to Ile$_{1726}$ followed by two TAA stop codons. (SEQ ID NOS: 4 AND 6) This construction gave rise to a recombinant protein which was anchored in the plasma membrane of infected cells by a glycosyl phosphatidyl inositol (GPI) type structure.

Figure 1C represents the PfMSP1$_{p19}$S recombinant protein sequence before cutting out the signal sequence. (SEQ ID NO: 7)

Figure 1D represents the PfMSP1$_{p19}$S recombinant protein sequence after cutting out the signal sequence. (SEQ ID NO: 9)

The amino acids underlined in Figures 1C and 1D originate from the EcoR1 site used to join the nucleotide sequences derived from the N-terminal portion of the MSP-1 of *P. vivax* (with signal sequence) and the MSP-1$_{p19}$ of *P. falciparum*.

Figure 2 - The soluble recombinant PfMSP1$_{p19}$ antigen purified by immunoaffinity was analyzed by immunoblot using SDS-PAGE in the presence (reduced) or absence (non reduced) of ß-mercaptoethanol. Samples w re charged onto gel after heating to 95°C in th presence of 2% SDS. Under these conditions only covalent type bonds (disulphide

bridges) can resist disaggregation. The left hand blot was revealed with a monoclonal antibody which reacted with a linear epitope of natural p19. The right hand blot was revealed with a mixture of 13 human antisera originating from subjects with acquired immunity to malaria due to *Plasmodium falciparum*. These results show that the recombinant baculovirus molecule can reproduce conformational epitopes in the form of a polymer the majority of which are recognized by human antiserum.

Figure 2B: Immunoblot analysis with human antiserum of recombinant purified MSP-1 p19 from *P. vivax* and *P. cynomolgi* under non reduced (NR), reduced only in the charging medium (R) and irreversibly reduced (IR) conditions:

This work was based on the idea that the baculovirus expression system correctly reproduced the conformational epitopes present *in vivo* on the C-terminal portion of MSP-1 in large amounts. The best means of measuring this property (which may be the only possible means in the absence of native purified proteins corresponding to p19) was to study the reactivity of the recombinant proteins with the antiserum of individuals exposed to malaria, this reflecting the native proteins as "seen" by the human immune system.

Thus soluble recombinant PvMSP-1 p19 and PcMSP-1 p19 antigens purified by immunoaffinity were analyzed by immunoblot using SDS-PAGE (15%) in the presence (reduced) or absence (non reduced) of DTT. Samples were loaded onto the gel after heating to 95°C in the presence of 2% SDS. The irreversible reduction was carried out as follows: the protein was resuspended in 0.2 M Tris-HCl, pH 8.4, 100 mM DTT, 1.0% SDS and heated for 30 minutes at 70°C. After diluting with water, acrylamide was added to a final concentration of 2 M and the mixture was incubated under nitrogen in th dark for 1 hour at 37°C. The immunoblot was revealed with a mixture of 25 human antisera originating from subjects with an acquired

immunity to malaria due to *Plasmodium vivax*. V and C respectively designate proteins derived from the MSP-1 of *P. vivax* and *P. cynomolgi*. It should be noted that irreversibly reduced recombinant proteins exhibited no reactivity with human antiserum while non irreversibly reduced proteins or non reduced proteins exhibited good reactivity. (The non reduced Pv MSP-1 p19 was a little weak since in its glycosylated state it does not bind well to nitro-cellulose paper). These results show that recognition of baculovirus MSP-1 p19 molecules by human antiserum is largely if not completely dependent on conformational epitopes sensitive to reduction which are reproduced in this system.

Figure 3 - The soluble $PvMSP1_{P42}$ recombinant antigen (Longacre et al., 1994, op. Cit.) was incubated for 5 hours at 37°C in the presence of protein fractions derived from merozoites of *P. falciparum* and separated by isolectrofocussing. The samples were then analyzed by immunoblot in the presence (reduced) or absence (non reduced) of ß-mercaptoethanol. Isolectrofocussing fractions 5 to 12, and two total merozoite extracts made in the presence (Tex) or absence (T) of detergent, were analyzed. The immunoblot was revealed with monoclonal antibodies specific for $MSP1_{p42}$ and $_{p19}$ of *P. vivax*. The results suggest that there is a proteolytic activity in the *P. falciparum* merozoites which can be extracted with detergent. Digestion of p42 in certain fractions appear to cause polymerization of the digestion products (p19); this polymerization is probably linked to the formation of disulphide bridges since in the presence of ß-mercaptoethanol, the high molecular weight forms disappear in favor of a molecule of about 19 kDa (Tex-R). The p19 polymerization observed in these experiments could thus be an intrinsic property of this molecule *in vivo*.

Figure 3B: The differential contribution of p42 and p19 antigens to the *P. vivax* anti-MSP-1 human response.

Recognition of *P. vivax* MSP-1 p42 and p19 antigens by the antiserum of individuals with an acquired immunity to *P. vivax* was compared using the ELISA inhibition technique as follows: a mixture of 25 human antisera originating from subjects with an acquired immunity to malaria due to *P. vivax* was diluted to 1:5000 and incubated for 4 hours at ambient temperature either alone, or in the presence of a 1 mM purified *P. vivax* recombinant p42 or p19. This mixture was transferred to a microtiter well which had been coated for 18 hours at 4°C with 500 $ng.ml^{-1}$ of purified absorbed recombinant p42 or p19, and incubated for 30 minutes at ambient temperature. After washing with PBS containing 0.1% of Tween 20, a goat anti-mouse IgG conjugated with peroxidase was added and the mixture was incubated for 1 hour at 37°C. The enzymatic activity was revealed by reading the optical density at 492 nm. The percentage inhibition was calculated based on values of 100% of antiserum activity with the coated antiserum on the microtiter plate in the absence of a competing antigen. Statistical data were calculated using a Statview program. Each bar represents the average percentage inhibition of a pair of competing/absorbed antigens based on 4 to 12 determinations; the vertical lines correspond to a 95% confidence interval. Asterisks (*) designate the antigens produced in the presence of tunicamycin, thus with no N-glycosylation. The important parameters of these measurements were the dilution of the antiserum by 1:5000 which is in the region which is sensitive to ELISA curves and the competing antigen concentrations of 1 mM which includes competition by low affinity epitopes. Thus these data reflect the maximum resemblance between the two compared antigens. The results show that the majority, if not all of the p42 epitopes recognized by the human antiserum are present on the p19 since in the presence of the latter, the reactivity of the human antiserum against p42 is inhibited as much as by the p42 antigen itself. In contrast, however, about 20% of the p19

epitopes recognized by human antiserum were not or were not accessible on the p42, since the reactivity of the human antiserum against the p19 was much less inhibited by p42 than by p19 itself. Such specific epitopes of p19 could be constituted or revealed only after cleaving the p42 into p19 and p33. These results were not affected by glycosylation showing that the effect is really due to a difference between the peptide components of p19 and p42 and not to a difference in glycosylation. These results underline the fact that p19 has a distinct immunological identity compared to p42.

Description of the PcMSP1$_{p19}$S (soluble) construction (soluble p19 of *P. cynomolgi*)

The DNA used for the above construction was obtained from a clone of the *Plasmodium cynomolgi ceylonesis* strain (22-23). This strain had been maintained by successive passages through its natural host (*Macaca sinica*) and cyclic transmissions via mosquitoes (27).

Blood parasites in the mature schizont stage were obtained from infected monkeys when the parasitemia had attained a level of 5%. They were then purified using the methods described in (25). The DNA was then extracted as described in (26).

A 1200 base pair fragment was produced using a PCR reaction using the oligonucleotides underlined in **Figure 4** originating from *P. vivax*. The 5' oligonucleotide comprised an EcoRI restriction site and the 3' oligonucleotide comprised two synthetic TAA stop codons followed by a BglII restriction site. This fragment was introduced by ligation and via these EcoRI and BglII sites into the pVLSV$_{200}$ plasmid already containing the signal sequence for the MSP-1 protein of *P. vivax* (19). The new plasmid (pVLSV$_{200}$C$_{42}$) was used to analyze the DNA sequences.

The *P cynomolgi* and the corresponding *P.vivax* sequences were aligned (SEQ ID NOS: 11-14). The black arrows designate the presumed primary and secondary cleavage sites. They wer determined by analogy with known sites in *P.*

falciparum (27, 28). The vertical lines and horizontal arrows localize the limits of the four regions which were studied. Region 4 corresponded to the sequence coding for the *P. cynomolgi* p19. Glycosylation sites are boxed and the preserved cysteines are underlined. The lower portion of **Figure 4** shows the percentage identity between the two isolates of *P. vivax* and *P. cynomolgi*.

The recombinant construction PcMSP1$_{p19}$S contains the DNA corresponding to 8 base pairs of the leader sequence and the first 32 amino acids of the MSP-1 of *Plasmodium vivax* from Met$_1$ to Asp$_{32}$ (Belem isolate; Del Portillo et al., 1991, P. N. A. S., 88, 4030) followed by GluPhe, due to the EcoR1 site, connecting the two fragments. This is followed by the sequence coding for the *Plasmodium cynomolgi* MSP1$_{p19}$ from Lys$_{276}$ to Ser$_{380}$ (Ceylon strain). The construction was terminated by two TAA stop codons. This construction gave rise to a recombinant protein which was secreted in the culture supernatant of infected cells.

Purification of recombinant PfMSP1p19 protein by immunoaffinity chromatography with a monoclonal antibody specifically recognizing the p19 of *Plasmodium falciparum*

The chromatographic resin was prepared by binding 70 mg of a monoclonal antibody (obtained from a G17.12 hybridoma deposited at the CNCM [National Collection of Microorganism Cultures] (Paris, France) on the 14th February 1997, registration number I-1846; this G17.12 hybridoma was constructed from X63 Ag8 653 myeloma producing IgG 2a/k recognizing the *P. falciparum* p19) to 3 g of activated CNBr-Sepharose 4B (Pharmacia) using standard methods detailed in the procedure employed by Pharmacia. The culture supernatants containing the soluble PfMSP1p19 were batch incubated with the chromatographic resin for 16 hours at 4°C. The column was washed once with 20 volumes of 0.05% NP40, 0.5 M of NaCl, PBS; once with 5 volumes of PBS and once with 2 volumes of 10 mM of sodium

phosphate, pH 6.8. Elution was carried out with 30 ml of 0.2 M glycine, pH 2.2. The eluate was neutralized with 1 M sodium phosphate, pH 7.7 then concentrated by ultrafiltration and dialyzed against PBS. To purify the anchored PfMSP1p19, all of the washing and elution solutions contained a supplemental 0.1% of 3-(dimethyl-dodecylammonio)-propane sulphonate (Fluka).

Recombinant *Plasmodium vivax* (p42 and p19) MSP1 vaccination test in the squirrel monkey *Saimiri sciureus*

This vaccination test was carried out on male non splenectomized 2 to 3 year old *Saimiri sciureus boliviensis* monkeys. Three monkeys were injected 3 times intramuscularly at 3 week intervals with a mixture of about 50 to 100 µg each of recombinant soluble $PvMSP1_{p42}$ and $_{p19}$ (19), purified by immunoaffinity. Complete and incomplete Freunds adjuvant was used as follows: 1st injection: 1:1 FCA/FIA; 2nd injection: 1:4 FCA/FIA; 3rd injection: FIA. These adjuvant compositions were then mixed 1:1 with the antigen in PBS. Five control monkeys received the glutathione-S-transferase (GST) antigen produced in *E. coli* using the same protocol. The challenge infection was carried out by injecting 2×10^6 red blood cells infected with an adapted *Plasmodium vivax* strain (Belem) 2.5 weeks after the final injection. The protection was evaluated by determining parasitemia daily in all animals by examining smears stained with Giemsa.

The curves in **Figure 5** show the variation in the measured parasitemia as the number of parasitic red blood cells per microliter of blood (logarithmic scale on the ordinate) as a function of the time passed after infection (in days). Curve A corresponds to the average values observed in the three vaccinated monkeys; curve B corresponds to the average values in the five control monkeys.

An examination of the Figure shows that the effect of th vaccination was to greatly reduce the parasitmisa.

Recombinant *Plasmodium cynomolgi* (p42 and p19) MSP1 vaccination test in the toque macaque *Macaca sinica*

Fifteen captured monkeys were used as follows: (1) 3 animals injected with 100 µg of soluble $PcMSP1_{p42}$; (2) 3 animals injected with 35 µg (1st injection) or 50 µg (2nd and 3rd injections) of soluble $PcMSP1_{p42}$; (3) 3 animals injected with a mixture of $PcMSP1_{p42}$ and $_{p19}$; (4) 3 animals injected with adjuvant plus PBS; (5) 3 animals not injected. Complete and incomplete Freunds adjuvant was used in the protocol described above. Injections were intramuscular at 4 week intervals. The challenge infection was made by injecting 2×10^5 red blood cells infected with *Plasmodium cynomolgi* 4 weeks after the last injection. Protection was evaluated by determining parasitemia daily in all animals by examining the parasitemia with Giemsa. Parasitemia were classified as negative only after counting 400 smear fields. The parasitemia were expressed as a percentage of parasitised red blood cells.

Figures 6A - 6G show the results obtained. Each of them shows parasitemia (expressed as the percentage of parasitised red blood cells along the ordinate on a logarithmic scale) observed in the challenge animals as a function of the time after infection (in days along the abscissa).

The results relate to:

- in **Figure 6A**; non vaccinated control animals;
- **Figure 6B** relates to animals which received a saline solution also containing Freunds adjuvant;
- **Figure 6C** is a superposition of figures 6A and 6B, with the aim of highlighting the relative results resulting from administration of Freunds adjuvant to the animals (the variations are clearly not significant);
  Figure 6D provides the results obtained after vaccination with p42;
- **Figure 6E** concerns animals vaccinated with p19 alone;

- finally, **Figure 6F** concerns animals vaccinated with a mixture of p19 and p42.

The p42 certainly induced a certain level of protection. However, as shown in Figures 6E and 6F, the protection conferred by the recombinant p19 of the invention was considerably better.

The numbers used to produce graphs (6A-6F) are given in **Figure 6G**.

***P. cynomolgi* toque macaque vaccination test; second challenge infection of monkeys vaccinated with p19 alone and controls (Figures 8)**

Six months later, with no other vaccination, the 3 macaques which received the p19 MSP-1 alone with FCA/FIA (Figure 6E) and the 3 macaques which received a saline solution containing Freunds adjuvant (Figure 6B) and 2 new unvaccinated monkeys underwent a new challenge infection by injecting 1 x 10^6 red blood cells infected with *Plasmodium cynomolgi*. Protection was evaluated by determining parasitemia daily in all animals by examining Giemsa smears. The parasitemia were classified negative only after counting 400 smear fields. The parasitemia were expressed as the percentage of parasitised red blood cells (the figures used to produce graphs 8A-C are given in **Figure 8D**). The six immunized animals which underwent challenge infection six months earlier had no detectable parasitemia except for 1 animal in each group which exhibited a parasitemia of 0.008% for 1 day (**Figures 8A and 8B**). The two unaffected controls exhibited a conventional parasitemia with a maximum of 0.8% and for 21 days (**Figure 8C**). Thus the 3 animals vaccinated with the MSP-1 p19 were as well protected six months later as the 3 controls which exhibited a complete conventional infection after the first challenge infection, despite th absence of or a very slight parasitemia after the first chall ng infection. (It is likely that protection against a homologous strain

of *P. vivax* in humans can also be induced by a single blood infection provided that the infection is allowed to run its natural course without treatment since untreated *P. vivax* infection in humans gave rise to complete clinical protection against the subsequent challenge with the identical strain but not with a different strain of the same species). Together these results suggest either that the protection period for p19 is at least six months or that the immunity induced can be effectively boosted by minimal exposure to parasitic infection.

Vaccination test with p19 in association with alum in the *P. cynomolgi* toque macaque system (Figures 9)

The previous positive protection results were obtained using complete (FCA) or incomplete (FIA) Freunds adjuvant. However, the only adjuvant which is currently allowed in man is alum. For this reason, we carried out a vaccination test with *P. cynomolgi* MSP-1 p19 in the toque macaque in the presence of alum as the adjuvant. Six captured macaques were used as follows: (1) 3 animals injected with 4 doses of 50 mg of recombinant *P. cynomolgi* MSP-1 p19 with 10 mg of alum; (2) 3 animals injected 4 times with physiological water and 10 mg of alum. The injections were intramuscular at 4 week intervals. The challenge infection was made by injecting 2×10^5 red blood cells infected with *P. cynomolgi* 4 weeks after the last injection. Protection was evaluated by daily determination of parasitemia in all animals by examining Giemsa smears. The parasitemia were classified negative only after counting 400 smear fields. Parasitemia were expressed as the percentage of parasitised red blood cells. The results of this experiment were as follows. 2 of the 3 macaques immunized with recombinant p19 with alum had about 30 times less total parasitemia during the infection period (**Figures 9A and 9B**) than the 3 control macaques immunized with physiological water and alum (**Figure 9D**) after the challenge infection. The third macaque immunized with p19 (**Figure**

9C) was not very different from the controls. For the vaccination test using *Plasmodium cynomolgi* p19 in the toque macaque, *macaca sinica*, described in Figure 9, the data used to produce the graphs (9A-9D) are given in (**Figure 9E**). While the results are a little less spectacular than the preceding results (Figures 6, 8), this is the first time that significant protection has been observed for recombinant MSP-1 with alum.

Figure 10: Vaccination test with a recombinant *Plasmodium falciparum* p19 in the squirrel monkey

Twenty *Saimiri sciureus guyanensis* (squirrel monkeys) of about 3 years old raised in captivity were used as follows: (1) 4 animals injected with 50 µg of soluble Pf MSP-1 p19 in the presence of Freunds adjuvant as follows: 1st injection: 1:1 FCA/FIA; 2nd injection: 1:4 FCA/FIA; 3rd injection: FIA. These adjuvant compositions were then mixed with 1:1 antigen in PBS; (2) 2 control animals received Freunds adjuvant as described for (1) with only PBS; (3) 4 animals injected with 50 µg of soluble Pf MSP-1 p19 in the presence of 10 mg of alum (Alu-Gel-S, Serva); (4) 2 control animals received 10 mg of alum with only PBS; (5) 4 animals injected with about 50-100 mg of GPI anchored Pf MSP-1 p19 reconstituted into liposomes as follows: 300 mmoles of cholesterol and 300 mmoles of phosphatidyl choline were vacuum dried and resuspended in 330 mM of N-octylglucoside in PBS with 1.4 mg of Pf MSP-1 p19, GPI. This solution had been dialyzed against PBS with adsorbent Bio-Beads SM-2 (Bio-Rad) and the liposomes formed were concentrated by centrifuging and resuspended in PBS The 1st injection was made with fresh liposomes kept at 4°C and the 2nd and 3rd injections were made with liposomes which had been frozen for preservation; (6) 2 animals injected with control liposomes made in the same way, in the absence of the p19, GPI antigen as described for (5); (7) 2 animals inject d with physiological water. Thre intramuscular injections were made at 4 week intervals. The challenge infection was made by

injecting 1×10^6 red blood cells infected with *Plasmodium falciparum*. Protection was evaluated by determining parasitemia daily in all animals by examining the Giemsa smears. Parasitemia were expressed as the percentage of parasitised red blood cells. The results of this vaccination test are shown in **Figures 10, A-G.**

The groups immunized with p19 in Freunds adjuvant or liposome demonstrated similar parasitemia to the control groups after a challenge infection (one animal (number 29) vaccinated with p19 in Freunds adjuvant died several days after challenge infection for reasons independent of vaccination (cardiac arrest). Irregularities in administration of the antigen in these 2 groups (poor Freunds emulsion, congealed liposomes) did not allow the significance of these results to be completely evaluated. In the alum group, 2 animals showed total parasitemia for the duration of the infection about 8 times less than the controls, 1 animal about 3 times less and 1 animal was similar to the controls. This experiment was a little difficult to interpret due to the variability in the controls, probably due to the strain of parasite used for the challenge infection which would not have been quite adapted to the non splenectomized *Saimiri* model developed only recently in Cayenne. However, the real effect with alum, although imperfect, is encouraging in that our antigens seem to be the only recombinant *P. falciparum* MSP-1 versions which currently have shown a certain effectiveness in combination with alum.

Figure 7: Specific reactivity with *P. falciparum* MSP1 p19 monkey anti-sera with high molecular weight aggregates (same antisera as for Figures 10)

Monkeys bred in captivity were injected intramuscularly with 1 ml of inoculum twice at 4 week intervals as follows: (1) 4 animals injected with 50 µg of soluble PfMSP1p19 in the presence of Freunds adjuvant as follows: 1st injection: 1:1 FCA/FIA; 2nd injection: 1:4 FCA/FIA; and mixed then 1:1 with the antigen in PBS; (2) 4 animals injected with 50 µg of soluble PfMSPp19 in the presence of 10 mg of alum; (3) 4 animals injected with about 50 µg of GPI anchored PfMSP1p19 reconstituted into liposomes composed of 1:1 molar cholesterol and phosphatidyl choline. The animals were bled 17 days after the second injection.

Red cells from a squirrel monkey with 30% parasitemia due to *P. falciparum* (with the mature forms in the majority) were washed with PBS and the residue was diluted 8 times in the presence of 2% SDS and 2% dithiothreitol and heated to 95° before being charged onto a polyacrylamide gel of 7.5% (separation gel) and 4% (stacking gel). After transfer to nitrocellulose, immunoblot analysis was carried out with antisera as follows: (1) pool of antisera of 4 monkeys vaccinated with soluble PfMSP1p19 in Freunds adjuvant, 1:20 dilution; (2) pool of antisera of 4 monkeys vaccinated with soluble PfMSP1p19 in alum adjuvant, 1:20 dilution; (3) pool of antisera of 4 monkeys vaccinated with anchored PfMSP1p19 in liposomes, 1:20 dilution; (4) monoclonal antibody, which reacts with a linear epitope of PfMSP1p19, 50 µg/ml; (5) SHI90 antisera pool originating from about twenty monkeys repeatedly infected with *P. falciparum* and which had become resistant to any subsequent infection with *P. falciparum*, 1:500 dilution; (6) antiserum pool of unaffected monkeys (never exposed to *P. falciparum*), 1:20 dilution.

The results in Figure 7 show that the 3 antisera pools of monkeys vaccinated with PfMSP1p19 either with Freunds adjuvant (track 1), alum (track 2) or in liposomes (track 3) reacted specifically with very high molecular weight complexes (diffuse in the stacking gel) and present in parasite extracts containing more mature forms. These results support the hypothesis that a specific aggregate of PfMSP1p19 is present *in vivo* comprising epitopes which are reproduced in recombinant PfMSP1p19 molecules synthesized in the baculovirus system, in particular oligomeric forms thereof.

In **Figure 7B**: The techniques and methods were the same as for Figure 7 except that the individual antiserum for each monkey was tested after three injections the day of the challenge injection and the SHI antiserum was diluted by 1:250. The numbers correspond to the individual monkeys noted in Figure 10. The results show that the antiserum for 4 monkeys vaccinated with p19 and alum reacted strongly and specifically with very high molecular weight complexes while the monkeys of other groups vaccinated with p19 and Freunds adjuvant or liposomes showed only a little reactivity as in the control reactivity with these complexes. Since the monkeys vaccinated with p19 and alum were also the best protected, this reactivity with the high molecular weight complexes appeared to indicate a protective effect, despite one monkey in the group not being protected with respect to the controls and that another was only partially protected.

The invention also, of course, concerns other applications, for example those described below with respect to certain of the examples, although these are not limiting in character.

Therapy

The recombinant molecule PfMSP1p19 can be used to produce specific antibodies which can possibly be used by passive transfer for therapeutics for severe malaria due to *P. falciparum* when there is a risk of death.

Diagnostics

The recombinant molecules PvMSP1p42, PvMSP1p19 and PfMSPp19 derived from baculovirus can and have been used to produce specific murine monoclonal antibodies. These antibodies, in combination with polyclonal anti-MSP1p19 antisera originating from another species such as the rabbit or goat can form the basis of a semi-quantitative diagnostic test for malaria which can distinguish between malaria due to *P. falciparum*, which can be fatal, and malaria due to *P vivax*, which is generally not fatal. The principle of this test is to trap and quantify any MSP-1 molecule containing the p19 portion in the blood.

In this context, the advantages of the MSP1p19 molecule are as follows:

(i) it is both extremely well conserved in the same species and sufficiently divergent between different species to enable specific species reactants to be produced. No cross reaction has been observed between antibodies derived from PfMSP1p19 and PvMSP1p19;

(ii) the function of MSP1p19, while not known with precision, seems to be sufficiently important that this molecule does not vary significantly or is deleted without lethal effect for the parasite;

(iii) it is a major antigen found in all merozoites and thus it must in principle be detectable even at low parasitemia and proportionally to the parasitemia;

(iv) since the recombinant MSP1p19 molecules derived from baculovirus appear to reproduce more of the native structure of MSP1p19, the

antibodies produced against these proteins will be well adapted to diagnostic use.

The microorganisms identified below have been deposited at the Collection Nationale de Culture de Microorganisms de L'Institut Pasteur (CNCM) under Rule 6.1 of the Treaty of Budapest on 1st February 1996, under the following registration numbers:

Identification reference	Registration numbers
PvMSP1p19A	I-1659
PvMSP1p19S	I-1660
PfMSP1p19A	I-1661
PfMSP1p19S	I-1662
PcMSP1p19S	I-1663

The microorganism identified below has been deposited at the CNCM under Rule 6.1 of the Treaty of Budapest on July 18, 1998 under the following registration number:

Identification reference	Registration number
P1MSP1p19S; His Ext; Sig Pf	I-2041

The invention also concerns the use of these antibodies, preferably fixed to a solid support (for example for affinity chromatography) for the purification of type p19 peptides initially contained in a mixture.

Purification means bringing this mixture into contact with an antibody, dissociating the antigen-antibody complex and recovering the purified p19 type peptide.

The invention also concerns vaccine compositions, also comprising mixtures of proteins or fragment, in particular mixtures of the type:

- *P. falciparum* p19 and *P. vivax* p19;
- *P. falciparum* p19 and *P. falciparum* p42, the latter if necessary being deprived of its most hypervariable regions;
- *P. vivax* p19 and *P. vivax* p42, the latter if necessary being deprived of its most hypervariable regions;
- *P. falciparum* p19 and *P. falciparum* p42, the latter if necessary being deprived of its most hypervariable regions, and *P. vivax* p19 and *P. vivax* p42, the latter if necessary being deprived of its most hypervariable regions.

In the present case, the most hypervariable regions are defined as region II or region II and all or part of region III, the portion of region III which is preferably deleted being that which is juxtaposed to region II (the conserved portion being located to the side of the C-terminal of p33, close to the p19). Regions II and III are illustrated in Figure 4.

The invention is not limited to the production of human vaccine. It is also applicable to the production of veterinary vaccine compositions using the corresponding proteins or antigens derived from parasites which are infectious for mammals and products under the same conditions. It is known that infections of the same type, babesiosis, also appear in cattle, dogs and horses. One of the antigens of the Babesia species has a high conformational homology (in particular in the two EFG-like and cysteine-rich domains) and functional homology with a protein portion of MSP-1 [(36), (37) and (38)].

Examples of veterinary vaccines using a soluble antigen against such parasites have been described (39).

It goes without saying that the p19s used in these mixtures can also be modified as described in the foregoing when considered in isolation.

The invention also concerns hybridomas secreting specific antibodies selectively recognizing the p19 of a MSP-1 protein in the merozoite form of a *Plasmodium* type parasite which is infectious for man other than *Plasmodium vivax* and which does not recognize *Plasmodium vivax*.

In particular, these hybridomas secrete monoclonal antibodies which do not recognize *Plasmodium vivax* and which specifically recognize *Plasmodium falciparum* p19.

The invention also concerns a hybridoma characterized in that it produces a specific antibody which specifically recognizes the p19 of *P. vivax* and the p19 of *P. cynomolgi*. A F10-3 hybridoma has been constructed from the X63 Ag8 653 myeloma producing IgG 2b/k recognizing the p42 glycoprotein of *Plasmodium vivax*. The F10-3 hybridoma has been deposited at the European Collection of Cell Cultures (ECACC) under Rule 6.1 of the Treaty of Budapest on August 6, 1998 under the Accession Number: 98080510.

It should be noted that in the text which follows as well as in the Figures whose numerals are headed by numbers 11, 12, 13, 14 and 15 respectively the references to $MSP1_{19}$ and $MSP1_{42}$ stand for p19 and p42 as they have been defined hereabove.

Of particular significance is the high degree of purity of the recombinant p19 proteins as obtainable by expression in baculovirus systems and immunoaffinity or metallo-affinity chromatography.

Particularly, the recombinant p19 protein is crystallizable implicating an absolute purity. In addition, mass spectrometry measurements also show a very high degree of purity estimated at greater than 95%.

Of particular significance also are the reduction-sensitive conformational epitopes in the two EGF domains of th preferred p19 recombinant proteins and the long term memory r sponse directed in a

substantially specific manner against said conformational epitopes, which said p19 recombinant proteins are capable of eliciting in laboratory animals.

These properties as well as other characteristics of preferred purified p19 recombinant proteins are further illustrated by the results provided by further studies which are reported hereafter.

The invention relates also particularly to recombinant proteins, as obtainable in a baculovirus vector system:

- in a pure state
- substantially free of any other form of recombinant protein which, has the same peptide sequences, but which contains alternate conformations in the two EGF regions. This alternate conformation is different from the conformational form as defined by:
- (a) the atomic coordinates as detailed in Annexes I, II or II obtained by crystallography (the Annexes I, II and II include respectively the atomic coordinates which define the *P. cynomolgi* MSP119, *P. vivax* MSP119, and *P. falciparum* MSP119 three-dimensional molecular structure); and
- (b) the NMR fingerprints as illustrated in Figures 12.0a to 12.2c.

The following data emphasize the importance of reduction-sensitive conformational epitopes in the two EGF domains comprising the MSP119 antigens. The quantitative reproduction of a single conformation likely to resemble the native parasitic protein in *Plasmodium* C-terminal MSP119 recombinant proteins derived from a higher order expression system such as baculovirus (indicated by crystal formation as described below), is thought to be one of the essential active principles necessary for the protective ff ct of th se antigens in the vaccination trials described previously. Thre types of experimental data are included:

(i) The spatial organization of the baculovirus recombinant MSP1$_{19}$ molecule from *Plasmodium cynomolgi* as defined by the determination of its crystal structure at 1.8 Å resolution. The probable structures of the corresponding MSP1$_{19}$ antigens derived from *Plasmodium vivax* and *Plasmodium falciparum* were determined by molecular modeling by the replacement of alternative residues present in the latter two species as compared to the *P. cynomolgi* MSP1$_{19}$.

(ii) The nuclear magnetic resonance (NMR) spectra for each of the 3 MSP1$_{19}$ recombinant proteins derived from *P. cynomolgi, P. vivax and P. falciparum.* These spectra represent precise, defined "fingerprints" of the conformation of the corresponding proteins in solution.

(iii) ELISA data indicating that the immune response to the recombinant MSP1$_{19}$ molecules is almost entirely directed against reduction-sensitive conformational epitopes both in humans exposed to the malaria parasite and in the highly protected toque monkeys inoculated with the *P. cynomolgi* p19 recombinant protein (Figure 6E). These results underline the importance of accurate and quantitative reproduction of these epitopes in the recombinant proteins designed to elicit a protective anti-malaria immune response. In addition it is shown that vaccination with the MSP1$_{42}$ recombinant protein favors a long term memory response which is preferentially directed against non-conformational epitopes present in the MSP1$_{42}$ in contrast to the MSP1$_{19}$ whose long term memory response is also directed against the conformational epitopes associated with protection.

Reference is made hereafter to the sets of drawings headed by numerals 11, 12, 13, 14 and 15, and to which the following legends respectively correspond.

Figure 11A: Backbone of $MSP1_{19}$ from *P. cynomolgi* showing disulfide bridges in bold line.

Figure 11B: Backbone of $MSP1_{19}$ showing positions of sequence differences between *P. cynomolgi* and P. vivax.

Figure 11C: Backbone of homology-modeled $MSP1_{19}$ of *P. falciparum* with positions of sequence differences with *P. cynomolgi*.

Figure 12: Reconstructed mass spectra and m/z spectra respectively of metalloaffinity purified *P. cynomolgi* (A,B), *P. falciparum* (C,D) and *P. vivax* (E,F) $MSP1_{19}$.

Figure 12.0a to 12.0c: Regions of the NOESY (a and b) and TOCSY (c) spectra of *P. cynomolgi* $MSP1_{19}$. The chemical shifts in the F1 and F2 dimensions are expressed in parts per million (ppm). (a) and (b): crosspeaks outside the diagonal correspond to dipolar interactions between two hydrogen nuclei of the protein. Signals at 4.67 ppm in (a) correspond to the residual water signal, and probably to dipolar interactions of H nuclei of the protein resonating at the same frequency as the water protons. (c): crosspeaks correspond to through-bond interactions between hydrogen nuclei in an amino acid residue. As in the NOESY spectrum, the peaks at 4.67 ppm arise from the residual water signal and probably from interactions of two nuclei in the same residue. Twenty positive contours are plotted at a vertical scale of 5000 (a, b) or 15000 (c) and a threshold level of 7 (a, b) or 6 (c) using the VNMR 5.3 software.

Figure 12.1: Regions of the NOESY (a and b) and TOCSY (c) spectra of *P. vivax* $MSP1_{19}$. Twenty positive contours are plotted at a vertical scale of 5000 (a), 4000 (b) or 30000 (c) and a threshold level of 7 (a, b) or 6 (c) using the VNMR 5.3 software.

Figure 12.2: Regions of the NOESY (a and b) and TOCSY (c) spectra of *P. falciparum* $MSP1_{19}$. Twenty positive contours are plotted at a vertical scale

of 5000 (a), 4000 (b) or 10000 (c) and a threshold level of 7 (a, b) or 6 (c) using the VNMR 5.3 software.

Figure 13: ELISA titration of tertiary monkey anti *P. cynomolgi* recombinant MSP1 antisera. Plates were coated either with native (N) or reduced, denatured (D) *P. cynomolgi* $MSP1_{19}$ or $MSP1_{42}$. OD; Optical density at 492 nm. Figures A to F represent respectively, monkeys 426-427-429 (anti- $MSP1_{19}$) and 428-434-435 (anti- $MSP1_{42}$) (Perera et al. 1998).

Figure 14: ELISA analysis of human *P. vivax* infected donors under reducing and non reducing conditions using immunoaffinity (A) or metallo affinity purified (B) $MSP1_{19}$ coating antigen.

Figure 15: ELISA titration of murine anti *P. cynomolgi* $MSP1_{19}$ and $MSP1_{42}$ antisera.

(I) Crystallization and structure determination

The expression of recombinant $MSP1_{19}$ in baculovirus is described by Holm et al., 1997 which is hereby incorporated by reference. Modified versions of the constructs described were produced by including a carboxyterminal hexahistidine tag to facilitate purification by metalloaffinity chromatography. Culture supernatants from spinner cultures were harvested and dialyzed extensively against 5 mM Tris-HCl, pH 8.0 at 4°C. The dialysate was adjusted to 20 mM Tris, pH 8.0, 0.1 M NaCl (loading buffer) and passed on columns of "Talon" metalloaffinity resin (Clontech, Palo Alto, U.S.A.) previously equilibrated with loading buffer. The charged resin was washed with loading buffer and eluted with the same buffer supplemented with 100 mM imidazole, pH 8.0. Eluted fractions were pooled and dialyzed against 10 mM potassium phosphate, pH 7.2.

Crystals were grown from a buffer containing 30% PEG 4000 (wt./vol.), 0.2 M ammonium acetate and 0.1 M sodium citrate at pH 5.6. The crystals belong to the trigonal space group $P3_221$ (the enantiomorph

P3121 was excluded during the structure analysis; see below) with unit cell dimensions a = b = 43.77 Å, c = 92.04 Å and with one molecule of MSP119 accommodated in the asymmetric unit.

All X-ray data were recorded with a MAR-Research image plate at the synchrotron beam line DW32, LURE, Orsay. Native diffraction data were obtained at 18°C from one crystal with dimensions 0.4 x 0.2 x 0.2 mm using an X-ray wavelength of 0.970 Å. A total of 139,331 Bragg intensities were measured between 15.0 to 1.8 Å resolution, which reduced to 9980 unique reflections upon merging equivalent reflections, corresponding to a complete data set within this resolution range. A single heavy-atom derivative was prepared by soaking a crystal overnight in a solution of p-chloromercuryphenyl sulphonic acid dissolved at a concentration of 6 mM in the crystallization buffer. Diffraction data were recorded at 18°C from one crystal, similar in size to that used as native, with an X-ray wavelength of 0.997 Å. The 57,824 individual intensity measurements reduced to a unique data set of 3905 reflections that was complete between the resolution limits of 20.0 and 2.5 Å. The unit cell dimensions of the derivative remained close to those of the native: a = b = 43.93 Å, c = 92.57 Å.

Isomorphous and anomalous difference Pattersons both gave peaks consistent with a single heavy-atom site. The mercury parameters were refined with the program SHARP (La Fortelle and Bricogne, 1997) using isomorphous and anomalous structure amplitude differences between the resolution limits of 13.0 and 2.5 Å. The figure of merit and phasing power was 0.56 and 1.7, respectively, for the acentric data, and 0.41 and 1.4, respectively, for the centric data. The correct space group enantiomorph was distinguished during phase refinement by solvent flattening with the program SOLOMON (Abrahams and Leslie, 1996); the final overall R-factor was 0.316 for P3121 and 0.223 for P3221, thus clearly indicating the latter space group.

The polypeptide chain could be readily traced for the whole of the first domain. By contrast, the second domain presented certain difficulties owing to lack of continuity of the electron density for some regions of the polypeptide chain. An electron density map offering a more facile interpretation of the second domain was subsequently obtained by combining the probability distributions of calculated phases from the traced structure of the first domain with those of SIRAS phases (using the program SIGMMA (Read, 1986)), followed by density modification of the resulting Fourier synthesis (using the program DM (Cowtan, 1994; Bayley, The CCP4 suite, 1994)). The atomic parameters were refined with the program REFMAC using the maximum-likelihood option. The final R-factor and free R-factor were 0.212 and 0.279, respectively, for all reflections included within the resolution limits of 15.0 to 1.8 Å.

The final model of $MSP1_{19}$ includes all residues from the amino terminus, Met1, to His91, the second residue of the carboxyterminal hexahistidine tag, with the exception of the segment from Asp66 to Asn68 located at the extremity of a ß-hairpin turn. In spite of continuous electron density being present in the region from Asp66 to Asn68, it was not sufficiently well defined to propose a conformation for the main chain of this segment, suggesting that this part of the structure is flexible. Although there was no unambiguous indication for multiple conformers for the side chains of $MSP1_{19}$ in the final electron density maps, the side chains of Lys50, Lys63 and Glu80 could not be modeled beyond their Cß atoms because of weak or absent density in these regions. The final model includes 65 solvent molecules. Annex I includes the atomic coordinates which define the *P. cynomolgi* $MSP1_{19}$ 3-dimensional molecular structure and Annexes II and III respectively are those of *P. vivax* and *P. falciparum* as determined by homology modeling.

Figure 11A shows the trace of the alpha-carbon backbone and disulfide bridges (bold lines) of the MSP119 molecule from *Plasmodium cynomolgi* determined by the solution of its crystal structure. The two epidermal growth factor (EGF)-like domains predicted from the primary structure are clearly visible as well as the characteristic disulfide bonds which are essential for the maintenance of this complex structure. It is important to note that although only 2 of the 3 "classic" EGF disulfide bonds are present in the first EGF domain for the *P. cynomolgi* and *P. vivax* moieties, the conformation of this domain does not differ significantly from either the second MSP119 EGF domain or other examples of EGF domains with 3 disulfide bridges.

One unexpected feature of the molecular structure of MSP119 is that the 2 EGF-like domains have close and extensive contacts at their interface which include a number of hydrophobic and polar interactions. An important implication of this observation is that the two EGF domains together form a very defined entity which is likely to be essential for the nature of the active protective principle in recombinant analogs of this molecule. These data suggest that the single EGF domains either alone or combined separately would not display the same required conformation.

The probable structures of the corresponding MSP119 antigens derived from *Plasmodium vivax* and *Plasmodium falciparum* (**Figures 11B** and **11C** respectively) were determined by molecular modeling based on the replacement of alternative residues present in the latter two species (11/89 for *P. vivax* and 48/89 + a 4-residue insertion for *P. falciparum)*. It is important to note that these replacements do not put any noticeable steric strains on the experimentally determined *P. cynomolgi* MSP119 structure and that the interactions at the EGF-domain interface are maintained by invariant residues or conservativ substitutions, in spite of several replacements seen particularly for the *P. falciparum* protein.

It should be emphasized here that, apart from allowing the precise determination of its molecular structure, the crystallization of the MSP119 recombinant protein is *prima facie* evidence of its absolute purity, its quantitatively reproducible, defined conformation as produced in the baculovirus expression system, and its stability. The fact that an identical preparation of *P. cynomolgi* MSP119 recombinant protein (as determined by mass spectrometry) was recently shown to confer excellent protection in the *P. cynomolgi*-toque monkey system described previously (Perera et al., 1998; S. Longacre, I. Holm, L. Perera and S. Handunetti, unpublished data) suggests that this particular conformation is important for protective efficacy.

(II) Nuclear magnetic resonance (NMR) "fingerprint" spectra

The three baculovirus MSP119 recombinant proteins derived from *P. cynomolgi, P. vivax* and *P. falciparum*, each with a carboxyterminal hexahistidine tag, were purified by metalloaffinity chromatography as described above, dialyzed extensively against 10 mM ammonium bicarbonate, and lyophilized. Five mg of lyophilized protein was dissolved in 380 ml of 20 mM deuterated sodium acetate, 10% D_2O, pH 4.0, centrifuged 30 min at 20°C and loaded into Shigemi (Shigemi Inc., Allison Park, P.A.) tubes.

In order to obtain an NMR "fingerprint" of a protein, the later must be pure to at least *ca.* 95%, as the signals of any proton-containing contaminant present at substantial concentrations would be observed in ^1H NMR spectra. The purity and sequence homogeneity of the MSP119 samples from *P. cynomolgi, P. falciparum* and *P. vivax* were assessed by electrospray mass spectrometry. The lyophilized prot in was dissolved in

water:methanol:formic acid (50:50:10). The sample was introduced in an API 365 triple-quadrupole mass spectrometer (Perkin Elmer-Sciex, Thornill, Canada) at 5 ml/min by means of a syringe pump (Harvard Apparatus, South Natick, MA). The device was equipped with an atmospheric pressure ion source used to sample positive ions which were produced by a pneumatically-assisted electrospray interface. The ion spray probe tip was held at 4.5 kV and the orifice voltage was set at 45 V. The mass spectrometer was scanned continuously from m/z 950 to 1600 (*P. cynomolgi* sample) or from 1050 to 1900 (*P. falciparum* sample) or from 1150 to 1800 (*P. vivax* sample) with a scan step of 0.1 and a dwell time per step of 2.0 ms. This resulted in a scan duration of 13.0 s (*P. cynomolgi* and *P. vivax* samples) and 17.0 s (*P. falciparum* sample). Ten scans were averaged for each experiment. Mass calibration of the instrument was accomplished by matching propylene glycol ions to their known reference masses which are stored in the mass calibration table of the spectrometer. Data were collected on a Power Macintosh 8600/200 and processed with the Biotoolbox 2.2 software from Sciex. The reconstructed mass spectra and m/z spectra of MSP119 from *P. cynomolgi*, *P. falciparum* and *P. vivax* are shown in **Figures 12A** and **B**, **12C** and **D**, and **12E** and **F** respectively. The spectra of all three proteins over a wide range of m/z values indicate that there is mainly a single species with no major contaminants, as all the major peaks correspond to different m/z values of a single species or its adducts. The average mass calculated from the experiment for the *P.cynomolgi* (10767 ± 1.1 Da), *P. vivax* (10524.70 ± 0.48) and *P. falciparum* (11041.1±0.6 Da) proteins correspond, within experimental error, to the expected mass of the oxidized proteins with an N-terminus at MSS (*P. cynomolgi* and *P. vivax)* and ISQ (*P. falciparum*). The minor peak in the *P. vivax* sample with an average mass of 10624.54 ± 0.70 corresponds to the expected mass of the same *P. vivax* protein with an N-

terminus at TMSS. Hence, the protein samples used for NMR experiments are greater than 95% pure as required to obtain an NMR "fingerprint" of the proteins and their conformation.

NMR experiments were carried out on a Varian Unity 500 spectrometer (11.7 T) operating at a proton frequency of 500 MHz and equipped with a 5 mm triple-resonance, z-gradient detection probe. Data were processed on a Sun workstation using the software VNMR 5.3 (Varian Inc., Palo Alto). All 2D proton NMR experiments were acquired in the phase-sensitive mode using the hypercomplex scheme (States et al., 1982). ^{1}H chemical shifts are referred to DSS (sodium 4,4-dimethyl-silapentane sulfonate) used as an external reference.

NOESY (States et al., 1982) and TOCSY (Griesinger et al., 1988) spectra were acquired at 35.0°C with 2048 data points in the direct dimension and 256 t_1 increments. Forty (*P. cynomolgi* NOESY), 48 (*P. cynomolgi* TOCSY), 64 (*P. vivax*) or 48 (*P. falciparum*) transients per t_1 increment were accumulated. Solvent suppression was achieved by using the WATERGATE pulse scheme (Piotto et al., 1992). Mixing times in NOESY and TOCSY were 120 and 70 ms, respectively. The spin lock during the mixing time of the TOCSY experiment was obtained by applying the MLEV-17 pulse sequence (Bax and Davis, 1985). Spectra were recorded using a spectral window of 6000 Hz and a recycling delay of 2.2 s (NOESY) or 2.0 s (TOCSY).

2D experiments were transformed using a low pass-filter centered at the transmitter frequency to reduce the intensity of the residual water signal. The width of the filter was 10 Hz and the number of coefficients to calculate the filter shape were 41. Spectra were apodized with shifted square sine bell functions in both dimensions, and baseline corrected in the direct dimension. The first point of every free induction decay (FID) was multiplied by 0.5 prior to Fourier transformation to reduce drift corrections.

The resolution in the indirect dimension (F1) was increased by forward linear prediction to 512 points, except for the TOCSY spectrum of the *P. vivax* sample. Zero filling to 4096 points in the direct dimension (F2) and to 2048 points in the indirect dimension was performed for each spectrum.

The chemical shift of a signal in an NMR spectrum depends on the chemical environment of the hydrogen nucleus that produces the signal. The chemical shift, and thereby the topology or peak pattern of an NMR spectrum, is highly dependent on molecular conformation. The TOCSY experiment gives through-bond connectivities within each amino acid residue of a protein while the NOESY experiment provides dipolar (through-space) connectivities between atoms that are less than 5 Å apart. The intensity of a signal strongly depends on the distance between two atoms, as well as on the dynamics of the molecule. The topology of a NOESY spectrum is thus particularly sensitive to differences in molecular conformation. Together, the TOCSY and NOESY spectra provide a detailed fingerprint of the structure of a given protein in solution.

The relevant regions of the NOESY (a and b) and TOCSY (c) spectra of the recombinant $MSP1_{19}$ from *P. cynomolgi* are shown in **Figure 12.0**. The good dispersion of the signals in both spectra is indicative of a folded protein. The down-field shift of many H resonances (**Fig. 12.0a and c**) relative to the random coil range (~4.1-~4.8 ppm) and the presence of strong sequential Hi-NHi+1 and weak NH-NH correlations in the NOESY spectrum (**Fig. 12.0b and a**, respectively) are all indicative of β-sheet structures and are consistent with the predominantly β-sheet structure determined for $MSP1_{19}$ by crystallography. The corresponding regions of the NOESY and TOCSY spectra of the recombinant $MSP1_{19}$ proteins from *P. vivax* and *P. falciparum* are displayed in **Figures 12.1** and **12.2**. As in the case of the *P. cynomolgi* protein, the spectra of both proteins show good dispersion of chemical shifts, down-field shifted H signals, strong

sequential Hi-NHi+1 and weak NH-NH correlations indicating that these proteins also have a substantial amount of β structures. As the proteins from *P. cynomolgi* and *P. falciparum* have *ca.* 50% different residues, a comparison of the NMR spectra of both proteins is not possible. However, the presence of β-sheet structures in *P. falciparum* $MSP1_{19}$ as evidenced by the NMR spectra, and the relatively high sequence identity of the proteins (*ca.* 48% for *P. cynomolgi* and *P. falciparum*) support their structural homology. The NOESY and TOCSY spectra of the *P. cynomolgi* and *P. vivax* proteins display many crosspeaks as well as crosspeak patterns at the same (or similar) chemical shifts. The similarities that can be observed in the spectra of both proteins, in spite of the existence of 11 different residues, suggest that both proteins have a similar structure. Thus, the NMR data, as well as the high sequence identity of both proteins (ca. 86%), strongly support the similitude of the $MSP1_{19}$ structures from *P. cynomolgi* and *P. vivax* species as postulated by homology modeling based on the crystallographic data.

Unlike X-ray crystallography, NMR technology provides a relatively accessible means of assessing if a protein preparation displays a given conformation. Thus, once having defined in detail a conformation which constitutes the active principle of a protective antigen, one can verify by NMR whether competing preparations contain conformationally similar species and/or whether these represent a mixture of more than one conformer (with an estimated 5-10% limit of detection). It should be noted that some variation in the peak position can be expected for independent experiments performed under the same conditions and with the same protein, with an estimated ±0.02 ppm variation in each dimension. Also, as the contour plots presented in **Figures 12.0, 12.1** and **12.2** are cross-sections of a spectrum which contains three-dimensional peaks as well as noise, and in which peak intensity dep nds on many experimental and

data-processing factors (such as the sensitivity of the probe, the suppression of the water signal, the phasing of the spectrum and the functions used to apodize the spectrum and correct the baseline) in addition to the nuclei interactions, some variations in the intensity and thereby in the pattern of crosspeaks at a given cross-section level must be expected, especially for the less intense peaks, the peaks near the diagonal and those close to the water signal (4.67 ppm). Bearing this in mind, the NMR spectra can serve as an identifying "fingerprint" which can be used not only for product quality control, but also to establish distinguishing characteristics of different preparations of the same or similar polypeptides.

(iii) ELISA analysis of anti-MSP1$_{19}$ and anti-MSP1$_{42}$ antibody reactivity with native and reduced antigen

Recombinant baculovirus *Plasmodium vivax* and *Plasmodium cynomolgi* MSP1$_{19}$ and MSP1$_{42}$ was produced and purified by immunoaffinity or metalloaffinity chromatography as described (Holm et al., 1997; see above). The antisera from *Macaca sinica* (toque) monkeys vaccinated with baculovirus recombinant *P. cynomolgi* MSP1$_{19}$ or MSP1$_{42}$ was obtained as described by Perera et al. (1998). Anti-*P. vivax* human antisera were obtained from endemically exposed donors in Thailand.

For the ELISA assay, microtiter plates (NUNC) were coated with the purified MSP1$_{19}$ or MSP1$_{42}$ recombinant protein at 200 ng ml^{-1} overnight at 4°C. After 3 washes with PBS containing 0.1% Tween 20 (PBS-T), the polyclonal human, monkey and mouse antisera were incubated for 2 h at 37°C. Plates were washed and incubated for 1 h at 37°C with horseradish peroxidase conjugated anti-human or anti-mouse IgG. After 5 washes, 100 µl of freshly prepared 0.2% orthophenylenediamine containing 0.03% hydrogen peroxidase in 0.1 M citrat buffer, pH 5.2 were added to each

well. The reaction was stopped by the addition of 50 µl of 3 N HCl and the optical density was measured at 492 nm. For ELISA assays done with reduced coating antigen, the antigen was reduced *in situ* by incubation in the microtiter plates with 0.2 M Tris-HCl (pH 8.5), 5 mM EDTA, 20 mM dithiothreitol for 2 h at room temperature followed by alkylation for 20 min in the dark with 60 mM iodoacetamide added to the reducing reaction mixture.

Figures 13 A-F show ELISA titration of tertiary antisera from 6 individual toque monkeys corresponding to 2 groups of 3 animals each vaccinated with either recombinant *P. cynomolgi* $MSP1_{19}$ or $MSP1_{42}$ (Perera et al. 1998). In each figure a given individual antisera is reacted with either the $MSP1_{19}$ or $MSP1_{42}$ antigen in native (N) or reduced, denatured (D) form. In the 3 antisera from $MSP1_{19}$ vaccinated animals (A-C) virtually all the epitopes in the $MSP1_{19}$ or $MSP1_{42}$ (also containing the $MSP1_{19}$ polypeptide) coating antigens are reduction sensitive since no significant ELISA reactivity is observed when the coating antigens are reduced. These results indicate clearly that the macaque humoral immune response to the recombinant $MSP1_{19}$ polypeptide, whether presented alone or in the context of the larger $MSP1_{42}$ protein, is primarily directed against reduction sensitive conformational epitopes. Since these $MSP1_{19}$ vaccinated monkeys were extremely well protected against a challenge infection of the *P. cynomolgi* blood stage parasite (Perera et al., 1998), it is thus highly likely that a conformationally intact recombinant $MSP1_{19}$ antigen is crucial for a relevant, protective immune response. The crystallographic results described above indicate that the $MSP1_{19}$ recombinant antigen obtained from this higher order expression system represents a single conformational species which must constitute, at least in part, the active principle in these vaccine preparations.

The antisera from MSP142 vaccinated monkeys (**Figures 13 D-F**) show that although all the MSP142 epitopes are not reduction sensitive, those corresponding to the MSP119 antigen are completely sensitive. From the above observations regarding the MSP119 vaccinated animals, it is likely that antibodies specific for the conformationally sensitive epitopes were important for the good protection conferred by the MSP142 antigen in 2 of the 3 vaccinated monkeys (n° 434 and 428; Perera et al., 1998). Nevertheless, the third animal (n° 435), which also had reduction sensitive anti-MSP119 antibodies, was not protected and it is possible that in some cases antibodies specific for non-conformational epitopes in the MSP142 immunogen could interfere with protection, arguing against the use of the MSP142 antigen in vaccine preparations.

Figure 14 shows the recombinant MSP119 reactivity of antisera (1:100 dilution) from 22 *P. vivax* infected Thai donors as determined by ELISA analysis using native or reduced antigen. The data from **Figures 14A** and **14B** were obtained using immunoaffinity or metalloaffinity purified antigens respectively. In all cases the native antigen is much more reactive than the reduced moiety, indicating that the large majority of MSP119 epitopes recognized by human anti-parasite sera are reduction sensitive. Thus any protective effect, as well as natural boosting, of an MSP119 based malaria vaccine would likely depend on the quantitative presence of such conformational epitopes in the vaccinating immunogen.

For **Figure 15**, 50 and 25 mg respectively of recombinant *P. cynomolgi* MSP142 and MSP119 purified by immunoaffinity chromatography (Holm et al., 1997) were inoculated subcutaneously three times into mice at 10 to 14 day intervals in the absence of adjuvant. A fourth and final boost was administered 8 months after the third injection. The dependence of the humoral immune response on reduction sensitive

conformational epitopes present in the recombinant antigens was analyzed with pooled anti-MSP142 or anti-MSP119 sera using the ELISA technique with native or reduced antigen as described above.

The results show that while the anti-MSP142 response is partially dependent on reduction sensitive conformational epitopes in the p42 moiety after 3 closely spaced injections (Third bleed), this dependence appears to be significantly reduced in the antisera obtained by boosting after an 8 month interval (Fourth bleed). Since the MSP142 antisera reactivity directed against MSP119 is completely dependent on reduction sensitive epitopes, the long term memory response to the MSP142 antigen in the absence of adjuvant clearly favors non-conformational, non-MSP119 epitopes. In contrast, the anti-MSP119 sera are completely dependent on reduction sensitive conformational epitopes regardless of the short or long term memory context. These observations, although preliminary, suggest that the N-terminal, non-p19 epitopes in the MSP142 might be favored by natural boosting to the detriment of the conformational MSP119 epitopes which appear to be important for protection (see above). This would also discourage the use of the MSP142 for vaccination under conditions where natural boosting by antigens containing MSP142 specific epitopes is expected.

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ANNEX I


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REMARK Wed May 13 11:19:38 1998
CRYST1   43.770   43.770   92.040  90.00  90.00 120.00
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ORIGX2      0.000000  1.000000  0.000000        0.00000
ORIGX3      0.000000  0.000000  1.000000        0.00000
SCALE1      0.022847  0.013189 -0.000001        0.00000
SCALE2      0.000000  0.026381 -0.000001        0.00000
SCALE3      0.000000  0.000000  0.010865        0.00000
REMARK      Atom Residue no.      X       Y       Z      Occ. B-fact  Z
ATOM      1  N   MET     1       1.213  13.847  37.031  1.00 51.49   7
ATOM      2  CA  MET     1      -0.202  14.188  36.715  1.00 51.41   6
ATOM      3  C   MET     1      -0.492  14.176  35.214  1.00 49.92   6
ATOM      4  O   MET     1       0.358  14.544  34.406  1.00 49.90   8
ATOM      5  CB  MET     1      -0.532  15.576  37.269  1.00 54.35   6
ATOM      6  CG  MET     1      -2.020  15.889  37.300  1.00 54.84   6
ATOM      7  SD  MET     1      -2.345  17.262  38.417  1.00 60.93  16
ATOM      8  CE  MET     1      -1.912  18.676  37.414  1.00 53.34   6
ATOM      9  N   SER     2      -1.730  13.837  34.870  1.00 46.47   7
ATOM     10  CA  SER     2      -2.160  13.819  33.477  1.00 44.34   6
ATOM     11  C   SER     2      -2.177  15.233  32.909  1.00 42.35   6
ATOM     12  O   SER     2      -2.584  16.178  33.584  1.00 38.52   8
ATOM     13  CB  SER     2      -3.526  13.156  33.364  1.00 47.86   6
ATOM     14  OG  SER     2      -4.433  13.932  32.606  1.00 51.95   8
ATOM     15  N   SER     3      -1.742  15.363  31.656  1.00 37.95   7
ATOM     16  CA  SER     3      -1.537  16.657  31.033  1.00 33.60   6
ATOM     17  C   SER     3      -2.810  17.465  30.900  1.00 27.50   6
ATOM     18  O   SER     3      -2.767  18.695  30.941  1.00 27.48   8
ATOM     19  CB  SER     3      -0.863  16.486  29.653  1.00 37.21   6
ATOM     20  OG  SER     3      -1.485  17.363  28.726  1.00 40.23   8
ATOM     21  N   GLU     4      -3.957  16.826  30.773  1.00 26.43   7
ATOM     22  CA  GLU     4      -5.229  17.497  30.655  1.00 24.35   6
ATOM     23  C   GLU     4      -5.537  18.349  31.905  1.00 22.72   6
ATOM     24  O   GLU     4      -6.288  19.309  31.806  1.00 20.76   8
ATOM     25  CB  GLU     4      -6.381  16.483  30.627  1.00 32.86   6
ATOM     26  CG  GLU     4      -6.937  16.258  29.223  1.00 39.74   6
ATOM     27  CD  GLU     4      -6.251  15.048  28.604  1.00 39.44   6
ATOM     28  OE1 GLU     4      -5.388  14.455  29.288  1.00 43.47   8
ATOM     29  OE2 GLU     4      -6.569  14.710  27.457  1.00 35.55   8
ATOM     30  N   HIS     5      -5.018  17.863  33.042  1.00 20.20   7
ATOM     31  CA  HIS     5      -5.365  18.609  34.277  1.00 18.01   6
ATOM     32  C   HIS     5      -4.302  19.574  34.743  1.00 21.28   6
ATOM     33  O   HIS     5      -4.466  20.228  35.796  1.00 19.19   8
ATOM     34  CB  HIS     5      -5.686  17.574  35.369  1.00 17.23   6
ATOM     35  CG  HIS     5      -6.924  16.767  35.111  1.00 16.21   6
ATOM     36  ND1 HIS     5      -8.204  17.256  35.259  1.00 18.48   7
ATOM     37  CD2 HIS     5      -7.031  15.467  34.737  1.00 16.39   6
ATOM     38  CE1 HIS     5      -9.066  16.286  34.989  1.00 19.00   6
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ANNEX II


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REMARK Fri Jul 10 10:11:56 1998
CRYST1   43.770   43.770   92.040  90.00  90.00 120.00
ORIGX1      1.000000  0.000000  0.000000        0.00000
ORIGX2      0.000000  1.000000  0.000000        0.00000
ORIGX3      0.000000  0.000000  1.000000        0.00000
SCALE1      0.022847  0.013189 -0.000001        0.00000
SCALE2      0.000000  0.026381 -0.000001        0.00000
SCALE3      0.000000  0.000000  0.010865        0.00000
REMARK      Atom Residue no.        X       Y       Z      Occ. B-fact   Z
ATOM      1  N    MET      1      1.213  13.847  37.031  1.00 51.49    7
ATOM      2  CA   MET      1     -0.202  14.188  36.715  1.00 51.41    6
ATOM      3  C    MET      1     -0.492  14.176  35.214  1.00 49.92    6
ATOM      4  O    MET      1      0.358  14.544  34.406  1.00 49.90    8
ATOM      5  CB   MET      1     -0.532  15.576  37.269  1.00 54.35    6
ATOM      6  CG   MET      1     -2.020  15.889  37.300  1.00 54.84    6
ATOM      7  SD   MET      1     -2.345  17.262  38.417  1.00 60.93   16
ATOM      8  CE   MET      1     -1.912  18.676  37.414  1.00 53.34    6
ATOM      9  N    SER      2     -1.730  13.837  34.870  1.00 46.47    7
ATOM     10  CA   SER      2     -2.160  13.819  33.477  1.00 44.34    6
ATOM     11  C    SER      2     -2.177  15.233  32.909  1.00 42.35    6
ATOM     12  O    SER      2     -2.584  16.178  33.584  1.00 38.52    8
ATOM     13  CB   SER      2     -3.526  13.156  33.364  1.00 47.86    6
ATOM     14  OG   SER      2     -4.433  13.932  32.606  1.00 51.95    8
ATOM     15  N    SER      3     -1.742  15.363  31.656  1.00 37.95    7
ATOM     16  CA   SER      3     -1.537  16.657  31.033  1.00 33.60    6
ATOM     17  C    SER      3     -2.810  17.465  30.900  1.00 27.50    6
ATOM     18  O    SER      3     -2.767  18.695  30.941  1.00 27.48    8
ATOM     19  CB   SER      3     -0.863  16.486  29.653  1.00 37.21    6
ATOM     20  OG   SER      3     -1.485  17.363  28.726  1.00 40.23    8
ATOM     21  N    GLU      4     -3.957  16.826  30.773  1.00 26.43    7
ATOM     22  CA   GLU      4     -5.229  17.497  30.655  1.00 24.35    6
ATOM     23  C    GLU      4     -5.537  18.349  31.905  1.00 22.72    6
ATOM     24  O    GLU      4     -6.288  19.309  31.806  1.00 20.76    8
ATOM     25  CB   GLU      4     -6.381  16.483  30.627  1.00 32.86    6
ATOM     26  CG   GLU      4     -6.937  16.258  29.223  1.00 39.74    6
ATOM     27  CD   GLU      4     -6.251  15.048  28.604  1.00 39.44    6
ATOM     28  OE1  GLU      4     -5.388  14.455  29.288  1.00 43.47    8
ATOM     29  OE2  GLU      4     -6.569  14.710  27.457  1.00 35.55    8
ATOM     30  N    HIS      5     -5.018  17.863  33.042  1.00 20.20    7
ATOM     31  CA   HIS      5     -5.365  18.609  34.277  1.00 18.01    6
ATOM     32  C    HIS      5     -4.302  19.574  34.743  1.00 21.28    6
ATOM     33  O    HIS      5     -4.466  20.228  35.796  1.00 19.19    8
ATOM     34  CB   HIS      5     -5.686  17.574  35.369  1.00 17.23    6
ATOM     35  CG   HIS      5     -6.924  16.767  35.111  1.00 16.21    6
ATOM     36  ND1  HIS      5     -8.204  17.256  35.259  1.00 18.48    7
ATOM     37  CD2  HIS      5     -7.037  15.467  34.737  1.00 16.39    6
ATOM     38  CE1  HIS      5     -9.066  16.286  34.989  1.00 19.00    6
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ANNEX III


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REMARK Wed May 13 12:12:21 1998
CRYST1   43.770   43.770   92.040  90.00  90.00 120.00
ORIGX1      1.000000  0.000000  0.000000        0.00000
ORIGX2      0.000000  1.000000  0.000000        0.00000
ORIGX3      0.000000  0.000000  1.000000        0.00000
SCALE1      0.022847  0.013189 -0.000001        0.00000
SCALE2      0.000000  0.026381 -0.000001        0.00000
SCALE3      0.000000  0.000000  0.010865        0.00000
REMARK      Atom Residue no.       X       Y       Z      Occ. B-fact  Z
ATOM      1  N   ASN     1        1.196  13.850  37.004  1.00 51.49   7
ATOM      2  CA  ASN     1       -0.208  14.191  36.732  1.00 51.41   6
ATOM      3  C   ASN     1       -0.469  14.171  35.223  1.00 49.92   6
ATOM      4  O   ASN     1        0.358  14.544  34.406  1.00 49.90   8
ATOM      5  CB  ASN     1       -0.523  15.583  37.280  1.00 54.35   6
ATOM      6  CG  ASN     1        0.685  16.519  37.262  1.00 54.84   6
ATOM      7  OD1 ASN     1        1.428  16.583  38.240  1.00 20.00   8
ATOM      8  ND2 ASN     1        0.930  17.259  36.196  1.00 20.00   7
ATOM      9  N   ILE     2       -1.728  13.838  34.876  1.00 46.47   7
ATOM     10  CA  ILE     2       -2.162  13.813  33.472  1.00 44.34   6
ATOM     11  C   ILE     2       -2.177  15.238  32.908  1.00 42.35   6
ATOM     12  O   ILE     2       -2.584  16.178  33.584  1.00 38.52   8
ATOM     13  CB  ILE     2       -3.559  13.197  33.374  1.00 47.86   6
ATOM     14  CG1 ILE     2       -3.621  11.765  33.903  1.00 20.00   6
ATOM     15  CG2 ILE     2       -4.082  13.133  31.937  1.00 20.00   6
ATOM     16  CD1 ILE     2       -5.017  11.149  33.796  1.00 20.00   6
ATOM     17  N   SER     3       -1.742  15.363  31.656  1.00 37.95   7
ATOM     18  CA  SER     3       -1.537  16.657  31.033  1.00 33.60   6
ATOM     19  C   SER     3       -2.810  17.465  30.900  1.00 27.50   6
ATOM     20  O   SER     3       -2.767  18.695  30.941  1.00 27.48   8
ATOM     21  CB  SER     3       -0.863  16.486  29.653  1.00 37.21   6
ATOM     22  OG  SER     3       -1.485  17.363  28.726  1.00 40.23   8
ATOM     23  N   GLN     4       -3.949  16.826  30.782  1.00 26.43   7
ATOM     24  CA  GLN     4       -5.246  17.504  30.650  1.00 24.35   6
ATOM     25  C   GLN     4       -5.528  18.342  31.901  1.00 22.72   6
ATOM     26  O   GLN     4       -6.288  19.309  31.806  1.00 20.76   8
ATOM     27  CB  GLN     4       -6.361  16.472  30.475  1.00 32.86   6
ATOM     28  CG  GLN     4       -7.128  16.639  29.163  1.00 39.74   6
ATOM     29  CD  GLN     4       -8.236  15.600  28.984  1.00 39.44   6
ATOM     30  OE1 GLN     4       -7.994  14.534  28.421  1.00 43.47   8
ATOM     31  NE2 GLN     4       -9.452  15.848  29.433  1.00 20.00   7
ATOM     32  N   HIS     5       -5.018  17.863  33.042  1.00 20.20   7
ATOM     33  CA  HIS     5       -5.365  18.609  34.277  1.00 18.01   6
ATOM     34  C   HIS     5       -4.302  19.574  34.743  1.00 21.28   6
ATOM     35  O   HIS     5       -4.466  20.228  35.796  1.00 19.19   8
ATOM     36  CB  HIS     5       -5.686  17.574  35.369  1.00 17.23   6
ATOM     37  CG  HIS     5       -5.924  16.767  35.111  1.00 16.23   6
ATOM     38  ND1 HIS     5       -8.204  17.256  35.259  1.00 18.48   7
```